ORIGINAL ARTICLE

ANTIPROLIFERATIVE EFFECTS OF A NOVEL MANGANESE MITOCHONDRIAL TARGETED COMPLEX

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Abstract

Curcumin, an active compound with multiple anti-cancer properties, is able to form organo-metallic complexes which exhibit superoxide dismutase like properties when complexed with manganese ions. The mitochondrial selectivity of a curcumin-manganese derivative in cancer cells could be accomplished by the addition of a triphenylphosphonium (TPP) radical. In this study, a TPP-manganese-curcumin complex was synthesized and assayed on three malignant human cell lines (melanoma, colon adenocarcinoma and osteosarcoma). Cell viability was evaluated and IC_{50} was determined for each cell line, including a control fibroblast line. For each cell line, the presence of manganese and TPP radical in the complex has improved the cytotoxic effects. Interestingly, the presence of TPP radical has reduced the cytotoxicity only for the normal fibroblasts. The leading mechanism for cytotoxic effects seems to be a redox one, since the presence of glutathione reduced the effects and inhibition of intracellular glutathione synthesis increased the toxicity.

Rezumat

Curcumina, o moleculă cu efecte antiproliferative, este capabilă să formeze complecși organo-metalici cu acțiune de tip superoxid dismutazic după complexarea cu ioni de mangan divalent. Selectivitatea mitocondrială pentru celulele canceroase a derivaților curcumină-mangan ar putea fi realizată prin adăugarea unui radical trifenilfosfoniu (TPP). Scopul acestui studiu a fost sinteza unui complex TPP-mangan-curcumină și analiza comparativă pe trei linii celulare maligne (melanom, adenocarcinom de colon și osteosarcom). Viabilitatea celulară și IC_{50} au fost determinate pentru fiecare linie celulară, constatându-se că prezența ionilor mangan și a radicalului TPP au îmbunătățit efectele citotoxice. Este de remarcat că prezența radicalui TPP a redus toxicitatea doar pentru fibroblaste. Mecanismul care conduce la efectele citotoxice descrise pare să fie de tip redox, deoarece prezența glutationului în mediu a redus efectele, iar inhibiția sintezei glutationului intracelular a crescut toxicitatea compușilor testați.

Keywords: curcumin, manganese, triphenylphosphonium radical, cytostatic potential

Introduction

Curcumin, an active phytochemical extract from *Curcuma longa*, has been used for centuries in a variety of pharmaceutical applications. The new compound proposed by this study was designed by modifying the curcumin structure, looking for three goals: redox properties improvement, chemical stability enhancement and targeting the mitochondria. Enhancing the redox properties of curcumin was achieved by metal complexation with divalent manganese cations. It is already reported that the complex manganese (II)-curcumin has strong antioxidant properties, being able to dismute the superoxide radical anions, thus having superoxide dismutase like properties [1].

On the other hand, metal complexation of curcumin could be a factor for increasing the chemical stability of curcumin, improving also the bioavailability of the final compound [2]. Improving the solubility by cyclodextrin complexation could be also a factor for the enhancement of the chemical stability.

The ability to target and to accumulate into mitochondria was achieved by using a triphenyl-phosphonium radical, connected through a n-butyl radical to one of the hydroxyl groups of curcumin. The triphenylphosphonium radical was chosen due to a good selectivity for mitochondrial membranes [3], together with a special selectivity for cancer cells mitochondria [4].

Despite a good scavenging activity, we expected to opposite effects if this complex could be able to accumulate in mitochondria, since a lot of antioxidant molecules are able to become pro-oxidant if a concentration threshold is induced [5, 6]. This behaviour is not uncommon, being already reported for other mitochondrial targeted SOD-

mimics [7]. Moreover, a potential selectivity for cancer cells mitochondria was expected, since many solid tumours have a more negative mitochondrial membrane potential compared to their normal counterparts [8].

Materials and Methods

Physical measurements

The elemental analyses of the synthesized complexes were performed on a 240 Perkin Elmer elemental analyser. The manganese content of the final complex was estimated using standard procedures after decomposing the complex with *aqua regia* followed by concentrated sulfuric acid.

The FT-IR spectra were recorded in KBr pellets on a DIGILABFTS 2000 spectrometer. ^{1}H-NMR spectra were recorded at room temperature on a Bruker Avance DRX-400 spectrometer (400 MHz) as solutions in dimethyl sulfoxide (DMSO), and chemical shifts are reported in ppm and referenced to tetramethylsilane TMS as internal standard. Spectral data (FTIR, NMR) were compatible with the final structure of the complex.

Chemical synthesis of TPP-manganese-curcumin

Curcumin (C) (1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione), (4-bromobutyl) triphenyl-phosphonium bromide, manganese (II) acetate were obtained from Sigma-Aldrich (USA).

Synthesis of Mn-C complex was performed according to literature [1]: briefly, 1.65 g of manganese (II) acetate in 10 mL ethanol was added dropwise to the solution of curcumin (2.4 g in 150 mL ethanol) and the mixture was allowed to reflux for 3 hours, at 60°C, under nitrogen gas. The final red-brown powder was filtrated and washed three times with cold ethanol.

Synthesis of TPP-Mn-C: A 60% dispersion of NaH (84 mg, 2.1 mmol) in oil was washed with pentane three times, and the solid residue dried in vacuum. Dry N,N-dimethylformamide (4 mL) was then added, and the resultant suspension was stirred for 15 min at room temperature. A solution of Mn-C (854 mg, 1.76 mmol) in dry N,N-dimethyl-formamide (6 mL) was then added dropwise *via* a cannula. The resultant solution was stirred for 90 min at room temperature, and a solution of (4-Bromobutyl)-tri-phenylphosphonium bromide (842 mg, 1.76 mmol) in N,N-dimethylformamide (6 mL) was then added dropwise. A brown precipitate formed, which was left under continuous mixing at room temperature for 48 h, under nitrogen gas. The final precipitate was filtrated and washed three times with cold ethanol.

IR (KBr) (cm^{-1}): 3402 (O-H), 2928 (alkene C-H), 2854 (alkane C-H), 1625 (C=O ketone), 1510 (aromatic C=C), 1260 (C-O), 1438 (aromatic C-H). ^{1}H NMR (d-DMSO): broad spectrum. Yield: 67.8%, Anal. Calc. for $C_{45}H_{48}BrMnO_8P$ (M.W. 882.67): C, 61.23; H, 5.48; Mn, 6.22. Found: C, 60.11; H, 5.26; Mn, 6.10.

Stock solutions of 10 mM curcumin (C) and curcumin derivatives (Mn-C and TPP-Mn-C) were prepared in DMSO and stored at -80°C.

Solubility evaluation

All the solubility tests were performed according to literature data [9], using different concentrations of complexes. The calibration curves were obtained by spectrophotometric analysis of solutions at 450 nm (the wavelength where both complexes present a maximum of absorption). Samples of complexes in quantities exceeding their solubility were ultra-sonicated for 10 minutes and shaken at room temperature for 72 hours. After filtration (0.22 μM Millipore filters), all the saturated solutions were spectrophotometrically analysed at 450 nm.

In order to increase the solubility of Mn-C and TPP-Mn-C, several cyclodextrins were tested (typical α, β and γ cyclodextrins, 2-hydroxypropyl-β-cyclodextrin and monochlorotriazinyl-β-cyclo-dextrin) and the best results were obtained by using monochlorotriazinyl-β-cyclodextrin (MCT-β-CD). After the addition of MCT-β-CD (2:1 molar ratio with the tested compounds) in DMSO, the solubility of the tested compounds increased about 1000 times. Testing MCT-β-CD alone in cell cultures showed no toxicity when the same concentrations were used. In solid form the compounds are stable for at least one month. When dissolved in DMSO, the biological activity decreased significantly after 24 hours, at room temperature.

Measurement of SOD-mimic proprieties

The SOD assay was carried out using a chemiluminescence method which measures the capacity of the tested compounds to inhibit the light emission from the system xanthine oxidase-allopurinol-lucigenin [10]. Briefly, 100 μL of micromolar solutions of Mn-C and TPP-Mn-C (made from the stock solutions by diluting with phosphate buffer saline (PBS) at pH 7.4) were automatically added and mixed with 700 μL allopurinol-xanthine oxidase-lucigenin system in PBS at pH 7.4: xanthine oxidase (8 mU/mL), allopurinol (125 μM), lucigenin (25 μM), Tween 20 (4.5 mM), catalase (300 U/ml) and EDTA (0.1 mM).

Cancer cell cultures ± glutathione (GSH)/ buthionine sulfoximine (BSO)

C, Mn-C and TPP-Mn-C were assayed at different concentrations (1–100 μM) following different conditions on three malignant cell lines: HOS (human osteosarcoma cells), HT 29 (human colon cancer cells), A375 (human melanoma cells). Human fibroblast cells (NHDF) were used as normal control. All the cell lines were purchased from Cell Lines Service CLS (Germany). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 2% sodium pyruvate, non-essential amino acids (2 mM), penicillin (100 units/mL),

streptomycin (100 mg/mL), and glutamine (4 mM). After achieving 70% confluence at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, cells were detached with trypsin-EDTA, counted and placed in 96-wells plates at a density of $2x10^4$ cells/well.

In order to prove the mechanism of action, the compounds were tested alone or in the presence of glutathione (GSH) and buthionine sulfoximine (BSO) a cellular glutathione synthesis inhibitor. Cells were depleted of glutathione (GSH) content by two hours treatment with 1 mmol/L buthionine sulfoximine (BSO, Sigma Chemical) dissolved in culture medium. Similarly, GSH levels in the cells were enhanced after treatment with GSH (5 mmol/L) for 2 hours, according to a published protocol [11].

MTT viability testing

Cellular viability was monitored by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test, as previously described [12]. After seeding cells in 96-well dishes, 48 hours of contact between cells and compounds at different concentrations were allowed. A MTT solution (0.3 mg/mL) was added to each well for 4 hours at 37°C. After removal of the supernatant, 200 µL DMSO was added and the absorbance was read at 570 nm. All assays were performed in triplicate.

Results and Discussion

Measurement of SOD-mimic properties

Using the allopurinol-xanthine oxidase-lucigenin chemiluminescence enhanced system, we tested four manganese compounds: Mn-C, TPP-Mn-C, $MnCl_2$ as a simple superoxide scavenger and EUK-8-[manganeseN,N'-bis(salicylidiene)ethylenediamine chloride] as a reference SOD-mimic. The percentages of superoxide inhibition were calculated at pH 7.4 and 10.1. The results demonstrated good superoxide anion scavenging activity for all the tested compounds (Table I) and only a minor decrease in activity was observed if the triphenylphosphonium radical was attached to the manganese curcumin complex.

Table I

SOD-like activity for the tested compounds, using the xanthine oxidase-allopurinol-lucigenin method

IC_{50} $MnCl_2$ (µM)	IC_{50} EUK-8 (µM)	IC_{50} Mn-C (µM)	IC_{50} TPP-Mn-C (µM)	
2.92 ± 0.2	1.16 ± 0.11	1.22 ± 0.12	1.29 ± 0.11	pH – 10.1
2.60 ± 0.16	1.19 ± 0.08	1.28 ± 0.14	1.36 ± 0.15	pH – 7.4

MTT viability testing

All the tested compounds (C – curcumin, Mn-C – manganese (II)-curcumin complex and TPP-Mn-C – n-butyl-triphenylphosphonium-manganese (II)-curcumin complex) showed antiproliferative effects for all the tested malignant cell lines (Table II). The presence of glutathione in medium (5 mM) leads to a reduced efficacy, while BSO (1mM) has the opposite effect. The cytotoxic effects of mitocurcuminoids were also assessed in normal human fibroblast cells (NHDF) and revealed a significant lack of toxicity for this type of cells when the TPP-Mn-Complex was used (IC_{50} > 100 µM).

Table II

IC_{50} values evaluated from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on four cell lines after 48 h exposure

		IC_{50} C (µM)	IC_{50} Mn-C (µM)	IC_{50} TPP-Mn-C (µM)
HOS	no additives	59 ± 3[a]	18.5 ± 1.2	19.6 ± 0.4
	+ GSH 5 mM	72.6 ± 4.1	56.9 ± 3.3	42.1 ± 2.4
	+ BSO 1 mM	4.4 ± 0.2	3.8 ± 0.1	3.7 ± 0.1
HT29	no additives	24 ± 1.1[b]	16.9 ± 0.8	9.8 ± 0.4
	+ GSH 5 mM	66.4 ± 3.6	46.1 ± 2.8	30.3 ± 1.6
	+ BSO 1 mM	4.8 ± 0.2	5.1 ± 0.2	4.3 ± 0.1
A375	no additives	50.1 ± 3.1[c]	16.3 ± 0.3	10.8 ± 0.4
	+ GSH 5 mM	> 100	20.1 ± 0.5	11.5 ± 0.5
	+ BSO 1 mM	10.1 ± 0.5	5 ± 0.1	4.7 ± 0.2
NHDF	no additives	51 ± 2.8	48 ± 2.6	> 100

HOS (human osteosarcoma cells), HT 29 (human colon cancer cells), A375 (human melanoma cells), NHDF (Human fibroblast cells) - consistent with literature data: [a] [13, 14], [b][15], [c][16].

Current results suggest that manganese complexation increased the cytotoxic effect for all the tested malignant cell lines, compared to the native curcumin. One possible explanation may consider the already reported redox properties of curcumin and the cytotoxic mechanism based on free radicals [17-19] which could be enhanced by the presence of manganese ions. Despite the proved antioxidant behaviour [20, 21], curcumin-manganese complexes could be able to act as prooxidant molecules if the concentration used is high enough. This hypothesis is supported by this study through the influence of glutathione or glutathione synthesis inhibitor, which are able to modulate in both ways the cytotoxicity of manganese complexes.

Another noteworthy result is related to the presence of the triphenylphosphonium radical and the main

benefit was a better selectivity, explained by the differences between the mitochondrial membrane potential of normal and malignant cells.
The presence of divalent manganese ions generated a significant decrease of IC_{50} for the manganese-curcumin complexes, especially for HOS and A375 cell lines.

Conclusions

In the present study, a new curcumin-manganese complex was synthetized and the potential anti-proliferative properties were investigated. As a concluding remark, the findings in this study provide another perspective regarding the development of metal-complexes which are able to target mitochondria and possess anti-cancer properties. Further in-depth investigations should be made in order to enhance even more the selective toxicity and stability of complexes and the results should be confirmed also in xenograft models.

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